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Angiopoietin correlates with glomerular capillary loss in anti-glomerular basement membrane glomerulonephritis

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Angiopoietin correlates with glomerular capillary loss in anti-glomerular basement membrane glomerulonephritis.

Background. Emerging evidence suggests that endothelial turnover occurs in several glomerular diseases and correlates with resolution or progression of glomerular lesions. We hypothesized that the growth factors modulating embryonic kidney endothelial cell survival and capillary morphogenesis may be implicated in capillary loss that occurs in immune-mediated glomerulonephritis (GN).

Methods. GN was induced in C57BL/6 mice by intravenous administration of sheep anti-mouse glomerular basement membrane (GBM) globulin and assessed with markers of vascularity in glomerular lesions, correlating these with expression of specific vascular growth factors.

Results. As assessed by periodic acid Schiff staining, 14 ± 4% (mean ± SD) glomeruli were affected by sclerosis at 14 days after globulin administration, and 33 ± 5% were affected at 21 days. By 21 days, a significant increase of plasma creatinine and urinary protein occurred. P-selectin expression was increased in glomerular capillaries 14 days after disease induction, and capillary loss, as assessed by immunohistochemistry for platelet-endothelial cell adhesion molecule, vascular endothelial growth factor (VEGF) receptor 2 and the angiopoietin (Ang) receptor Tie-2, was recorded at 14 and 21 days in glomeruli affected by proliferative crescents and/or sclerosis. VEGF-A immunostaining, evident in control glomeruli, was qualitatively diminished in glomeruli with lesions. Ang-1 immunostaining was detected in control glomeruli and was diminished at 14 days after administration of anti-mouse GBM globulin; instead, Ang-1 was immunolocalized to distal tubules. In contrast, Ang-2 immunostaining was barely detectable in control glomeruli but was prominent in disease glomeruli. In GN mice, rare apoptotic glomerular endothelia were detected by electron microscopy and in situ end-labeling, but such cells were not seen in controls.

Conclusions. Loss of glomerular capillaries during the course of anti-GBM GN in mice was temporally associated with decreases in endothelial survival molecules VEGF-A and Ang-1, and with up-regulation of Ang-2, an antagonist of Ang-1. A changing balance of these growth factors may contribute to decreased glomerular vascularity in crescentic GN.

Key words: progressive renal disease, glomerular lesions, capillary morphogenesis, vascular endothelial growth factor, Tie-2, sclerosis, crescentic glomerulonephritis.

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Each adult mammalian kidney receives 10% of the cardiac output, a high blood flow supplying glomerular and peritubular capillaries. Growth factors signaling through receptor tyrosine kinases (RTK) direct embryonic endothelial differentiation [1]. One set of RTK is the vascular endothelial growth factor receptors (VEGFR), with VEGFR-2 mediating endothelial formation and VEGFR-1 modulating vessel assembly [1]. These genes are expressed in differentiating metanephric mesenchyme and during later nephrogenesis [2–4], and functional experiments implicate VEGF-A in glomerular growth in vivo [5, 6] and in endothelial growth in metanephric organ culture [7].

Tie genes constitute another class of endothelial RTK [1]. As endothelia differentiate, the onset of Tie expression postdates VEGFR-2 but precedes maturity; platelet-endothelial cell adhesion molecule (PECAM), is expressed with Tie-2 during this process [4, 8]. *Tie* genes are implicated in differentiation of cells that have entered the endothelial lineage and, indirectly, also in reciprocal signaling from endothelia to maturing pericytes and vascular smooth muscle [1]. *Tie-1* null-mutants have impaired vessel integrity and, in $Tie\text{-}1^{lcz}/Tie\text{-}1^{lczn-}$ chimeras, mutant cells fail to contribute to renal vasculature, suggesting a nephrogenic role for Tie-1 [9]. Angiopoietins (Ang) contain an aminoterminal coiled-coil domain mediating the formation of dimers and multimers, with carboxyterminal fibrinogen-like domains mediating differential effects on Tie-2 phosphorylation [10]. Ang-1 binds to and tryosine phosphorylates Tie-2, preventing endothelial apoptosis and inducing capillary morphogenesis [11–13], and Ang-1 also reduces capillary plasma leakage during health and leukocyte transmigration in inflammatory states by stabilizing PECAM and inhibiting cytokine-induced leukocyte transmigration [14, 15]. Ang-2 inhibits Ang-1–induced Tie-2 phophorylation

[16], although a single report showed that, at very high concentration and prolonged exposure, Ang-2 stimulates Tie-2 [17]. By destabilizing existing vessels, Ang-2 facilitates vascular growth when ambient VEGF is high, but when VEGF is low, Ang-2 expression causes capillary loss, for example, in corpus luteum angiogenesis and regression [16, 18]. Angiopoietins and Tie receptors genes are expressed in nephrogenesis and Ang-1 enhances glomerular vascularization in metanephric organ culture [2, 4, 19–21]. As assessed by Ang-2 driven LacZ expression, Ang-2 is expressed in differentiating arterial smooth muscle and transiently in the mesangial lineage, with hypoxia up-regulating Ang-2 in cultured mouse mesangial cells [21, 22].

It has been reported that glomerular endothelial cell regression, occurring at least partly by apoptosis, is a feature of certain experimental models of glomerular disease, including the remnant kidney [23] and immune-based glomerulonephritis (GN) [24]. Furthermore, molecular signaling systems controlling nephrogenic endothelial growth are implicated in glomerular and peritubular capillary remodeling that occurs in animal models, such as acute neprotoxicity induced by folic acid [25], experimental thrombotic microangiopathy [26] and the remnant kidney [27, 28]. We hypothesized that growth factors modulating embryonic kidney endothelial cell survival and capillary morphogenesis also may be implicated in capillary loss in immune-initiated glomerular inflammation associated with leukocyte-dependent proliferative and crescentic GN [29]. We demonstrate that loss of glomerular capillaries in a mouse model of anti-glomerular basement membrane (GBM) induced GN is associated with decreases in endothelial survival molecules VEGF-A and Ang-1, and up-regulation of Ang-2, an Ang-1 antagonist. We postulate that a changing balance of these growth factors may contribute to decreased glomerular vascularity in this type of GN.

METHODS

General materials and antibodies

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). Antibodies used were: rabbit antibody to a 20 aa sequence in the N-terminal of mouse Ang-1 (Alpha Diagnostic International, San Antonio, TX, USA) for immunohistochemistry (1:500) and Western blot (1:1000); rabbit antibody to a 20 aa sequence in the N-terminal of mouse Ang-2 (Alpha Diagnostic International) for immunohistochemistry (1:500) and Western blot (1:1000) (note that Ang-1 and Ang-2 immunizing peptides have no significant homology); rat anti-mouse PECAM (Pharmingen, San Diego, CA, USA) for immunohistochemistry (1:1000); rat anti-mouse Tie-2 (a gift from Toshio Suda, Kumamoto University School of Medicine, Japan) [4, 25] for immunohistochemistry (1:500); rabbit anti-human VEGF-A (A-20; Santa Cruz) for immunohistochemistry (1:400) and Western blot (1:400); and rat antimouse VEGFR-2 (Pharmingen) for immunohistochemistry (1:1000). Relevant second antibodies were purchased from DAKO (Cambridge, Cambridgeshire, UK).

Induction of GN

Anti-glomerular basement membrane glomerulonephropathy was induced as described [29]. C57BL/6 strain mice were maintained under pathogen-free conditions at Monash University (Clayton, Victoria, Australia). Sheep anti-mouse GBM globulin (1.25 mg/10 g body wt) was administered intravenously to 12-week-old male mice. Mice were studied at 14 and 21 days after the administration of anti-GBM globulin, at which times the proliferative and sclerotic GN lesions are becoming morphologically established [29]. Mice that had received no globulin were used as controls. Preliminary analyses revealed no differences in any measured parameter in control mice at 14 versus 21 days, and hence, data sets from these times were amalgamated. Mice were housed individually in cages to collect urine over the final 24 hours of each experiment, and mice were bled under diethyl ether anesthesia (Unilab, Sevenhills, NSW, Australia). Immediately after death of each mouse, one kidney was removed for histology while the cortex of the contralateral organ retained for RNA and protein analyses. For convenience, in this article animals that did not receive anti-GBM globulin are referred to as the 'control' group, whereas those that received the anti-GBM globulin are the 'experimental' group.

Histology

Kidneys were fixed in Bouin's fixative, embedded in paraffin and sectioned at 4 μm. The following stains were performed: hematoxylin and eosin (H&E) for general histology and periodic acid-Schiff (PAS) for assessment of glomerular sclerosis and detection of proximal tubule brush borders. Glomeruli were examined at their approximately maximum diameter (that is, equatorial cross sections) and were considered to exhibit crescent formation when three or more layers of cells were detected in Bowman's space. For immunohistochemistry, deparaffinized sections were pre-treated with proteinase (100 μg/mL), proteinase K (100 μg/mL) or trypsin (1.0 mg/mL) for 10 to 25 minutes, depending on the primary antibody. Endogenous peroxidase was quenched with 3% hydrogen peroxide in water for 30 minutes and sections were blocked in 10% bovine serum with 0.1% Tween-20. After incubation sequentially with primary antibodies and biotin-conjugated secondary antibodies, bound antibodies were detected with a streptavidin-biotin peroxidase system or Envision kit (Dako, Hamburg,

Germany). Controls included omission of the primary antibodies or pre-incubation of primary antibodies with blocking peptides. Staining of PECAM and in situ end-labeling was performed to detect apoptotic endothelia. Sections were pretreated with proteinase K for 15 minutes and reaction mixture was added and incubated at 37°C for two hours. After TUNEL staining using a fluorescein isothiocyanate detection method (TUNEL kit; Boehringer Mannheim, Lewes, East Sussex, UK), sections were incubated with rat anti-mouse PECAM, and then rabbit anti-rat Ig conjugated with Texas Red (1:100; Dako) was used as secondary antibody. Sections were mounted with aqueous mounting medium (Citiflour Ltd, London, UK) and examined by confocal microscopy. For electron microscopy, 100 μm thick paraffin sections were dewaxed and rehydrated and then fixed in 3% glutaraldehyde in pH 7.4 0.1 mol/L Na cacodylate and 5 mmol/L NaCl. Ultrathin sections were cut on an RTC MT6000 ultramicrotome using a Diatome diamond knife (Agar Scientific Ltd, Stansted, UK). Sections were stained with 25% uranyl acetate in 50% methyl alcohol and Reynold's lead citrate, each for 20 minutes and grids examined with a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan).

Northern blotting

Total RNA was isolated with Tri-Reagent and 20 μg was electrophoresed in 1% formaldehyde-denatured agarose gel in 1× MOPS buffer, transferred onto Hybond-N membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and fixed with UV-Stratalinker (Stratagene, La Jolla, CA, USA). Equality of loading was confirmed by visualizing 18S rRNA. Ang-1, Ang-2, Tie-2 and VEGF-A mouse cDNA plasmids were used for hybridization, as described [20, 22]. Inserts were isolated with appropriate restriction enzymes and random primer labeling was performed with Prime-a-Gene labeling system (Promega, Southampton, UK). Unincorporated labeled-dCTP was removed by using a push-column (Stratagene). Blots were prehybridized with Quick-Hyb solution (Stratagene) at 65°C for 30 minutes and hybridized with specific probes at 65°C for two hours. After hybridization the filters were washed twice with 2× standard sodium citrate (SSC) at 65°C for 30 minutes and once with 0.1× SSC / 0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 minutes. X-ray films were exposed to blots for 24 to 72 hours at −80°C.

Western blotting

Kidneys were homogenized in RIPA buffer at 4°C and the supernatants were collected by centrifugation at 13,000 rpm for 30 minutes. Supernatants were used for protein determination (BCA protein assay; Perbio Science UK Ltd, Tattenhall, Cheshire, UK). For Western blotting, 30 μg of protein samples were boiled for five minutes and separated on 6 to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) by electroblotting (BioRad). Blots were blocked overnight at 4°C with 5% (wt/vol) fat-free milk powder, 0.2% bovine serum albumin (BSA) and 0.3% (vol/vol) Tween-20 in PBS and then incubated with appropriate primary antibodies overnight at 4°C. After washing in blocking solution, blots were incubated for 30 minutes with horseradish peroxidase-conjugated second antibodies in blocking solution. Blots were washed three times with blocking solution and once with phosphate-buffered saline (PBS). Bound antibodies were detected using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (Amersham Pharmacia Biotech). Proteins were sized with Rainbow markers. Some blots were reprobed after the antibody complex was stripped using striping buffer (Chemicon, Harrow, UK).

Statistics

Glomeruli containing apoptotic cells were quantified as follows. Two histological cross-sections of whole kidneys, separated by at least 100 μm to avoid counting the same glomeruli twice, were analyzed from four kidney samples in each group after double-staining with PECAM antisera and TUNEL reagent, as described above. For each kidney, glomeruli in both sections were pooled, and the following was noted: (*1*) the total number of glomeruli, (*2*) the percentage of these glomeruli which contained one or more TUNEL-positive cells, (*3*) the percentage of glomeruli containing cells that were TUNEL-positive and also expressed PECAM protein, and (*4*) the percentage of glomeruli containing one or more cells that were TUNEL-positive but not immunoreactive with PECAM antibody. Values were expressed as means ± SEM. One-way ANOVA and the Bonferroni Post Hoc test are used to test the statistical differences and $P < 0.05$ was considered statistically significant. Intensities of Northern blot and Western blot signals were respectively measured relative to 18S rRNA signals or G Brilliant blue staining with an image densitometry analysis program (Phoretix 1D, Newcastle upon Tyne, UK), with the mean of the control group was designated as an arbitrary value of 1. Four samples were used in each data set for Northern blot and three samples for Western blot. For quantification of histological data, all glomeruli were examined in one cross-section of each kidney (representing 99 to 193 glomeruli per sample), and the results (for example, for glomeruli with PAS-positive deposits or apoptosis) expressed as percentages. Group data were compared by the Turkey test, with $P < 0.05$ considered statistically significant.

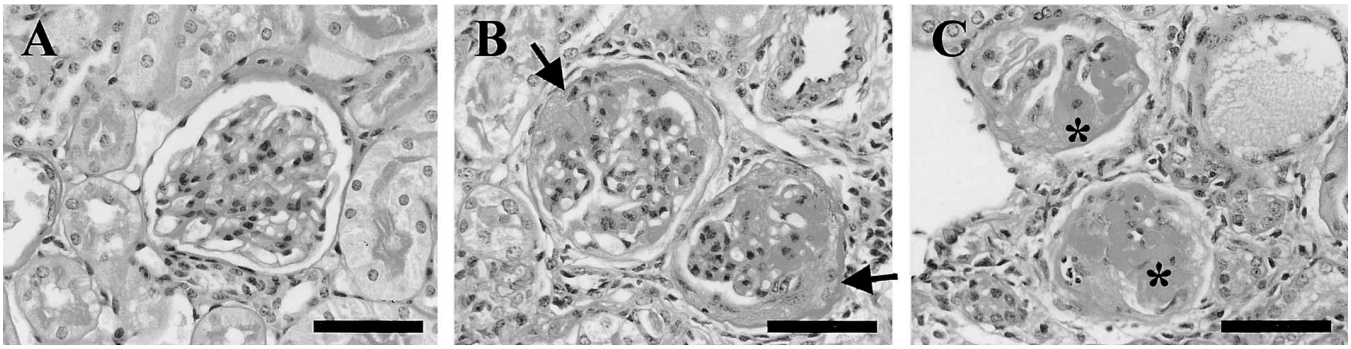

Fig. 1. Glomerular morphology. Sections stained with PAS and hematoxylin. (*A*) Glomerulus from control kidney. (*B*) Cellular crescents (arrows) in two glomeruli on experimental day 14. (*C*) Sclerotic glomeruli (*) from kidney on experimental day 21. Bars are 80 μm.

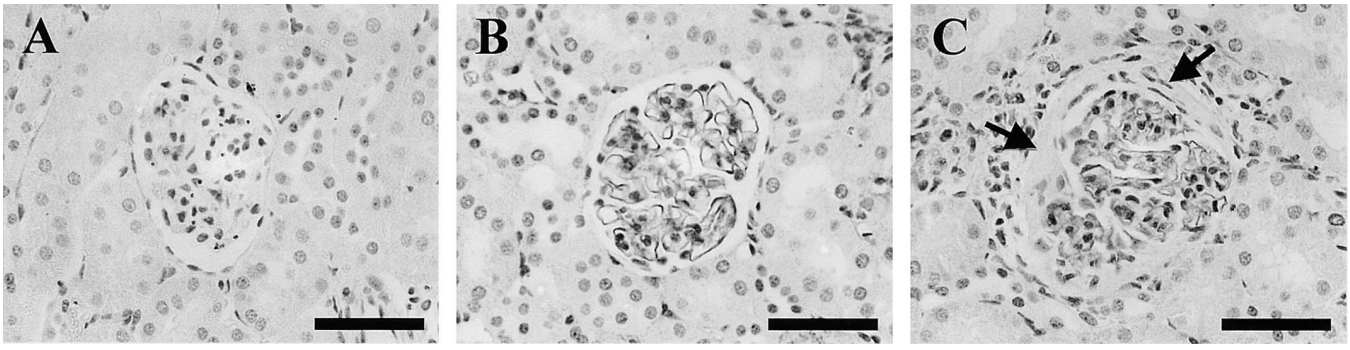

Fig. 2. P-selectin immunohistochemistry. All sections stained with hematoxylin and reacted with anti-P-selectin antibody. (*A*) Glomerulus from control kidney shows faint immunostaining (brown). (*B*) Prominent immunoreactive P-selectin in a morphologically normal glomerulus from anti-GBM GN kidney. (*C*) Crescentic lesion (arrows) in a glomerulus from anti-GBM kidney shows absent immunostaining while P-selectin is expressed in the intact portion of the tuft. Bars are 80 μm.

RESULTS

Induction of anti-GBM GN

Protein excretion (mean ± SD) in control mice was 3.0 ± 1.1 mg/24 h ($N = 4$). Fourteen days after administration of anti-GBM globulin, there was a non-significant increase of proteinuria (4.9 ± 1.9 mg/24 h; $N = 8$). At day 21 after induction of disease, urine protein excretion was significantly ($P < 0.01$) increased (10.0 ± 3.8 mg/24 h; $N = 8$). Plasma creatinine concentration in control mice was 25.9 ± 6.3 μmol/L ($N = 4$). Fourteen days after administration of anti-GBM globulin, there was a non-significant increase of plasma creatinine (38.6 ± 7.8 μmol/L; $N = 8$). At day 21 after induction of disease, plasma creatinine was significantly ($P < 0.05$) increased in the experimental group (43.7 ± 11.9 μmol/L; $N = 8$). As assessed by histology (Fig. 1), after administration of anti-GBM globulin, mice developed a predominantly focal and segmental GN. Cellular crescents predominated at 14 days (Fig. 1B), with sclerotic glomerular lesions more prominent at 21 days (Fig. 1C). For example, on day 14, there were 14.4 ± 4.3% of glomeruli ($N = 4$ kidneys) containing PAS-positive segmental lesions, and by experimental day 21 this number had significantly ($P < 0.01$) increased to 33.3 ± 5.4% ($N = 4$ kidneys). Such lesions were not observed in kidneys of control mice.

Detection of glomerular capillaries

P-selectin, is a marker of endothelial activation [30] and was barely detectable using immunohistochemistry in glomeruli of control kidneys (Fig. 2A). By contrast, prominent P-selectin immunostaining was noted 14 days after administration of anti-GBM globulin in >90% of glomeruli, whether these were histologically normal (Fig. 2B), or affected by morphological GN (Fig. 2C). In glomeruli with morphological signs of GN at 14 and 21 days after anti-GBM globulin, P-selectin immunostaining was decreased or absent within the cellular and sclerotic lesions (Fig. 2C). Glomerular capillaries were further visualized using a battery of endothelial markers: PECAM, VEGFR-2 and Tie-2. In all glomeruli from control animals and also in morphologically normal glomeruli from experimental animals, capillaries were distributed evenly across glomerular equatorial cross-sections (Fig. 3 A-C). In contrast, immunostaining for these endothelial mark-

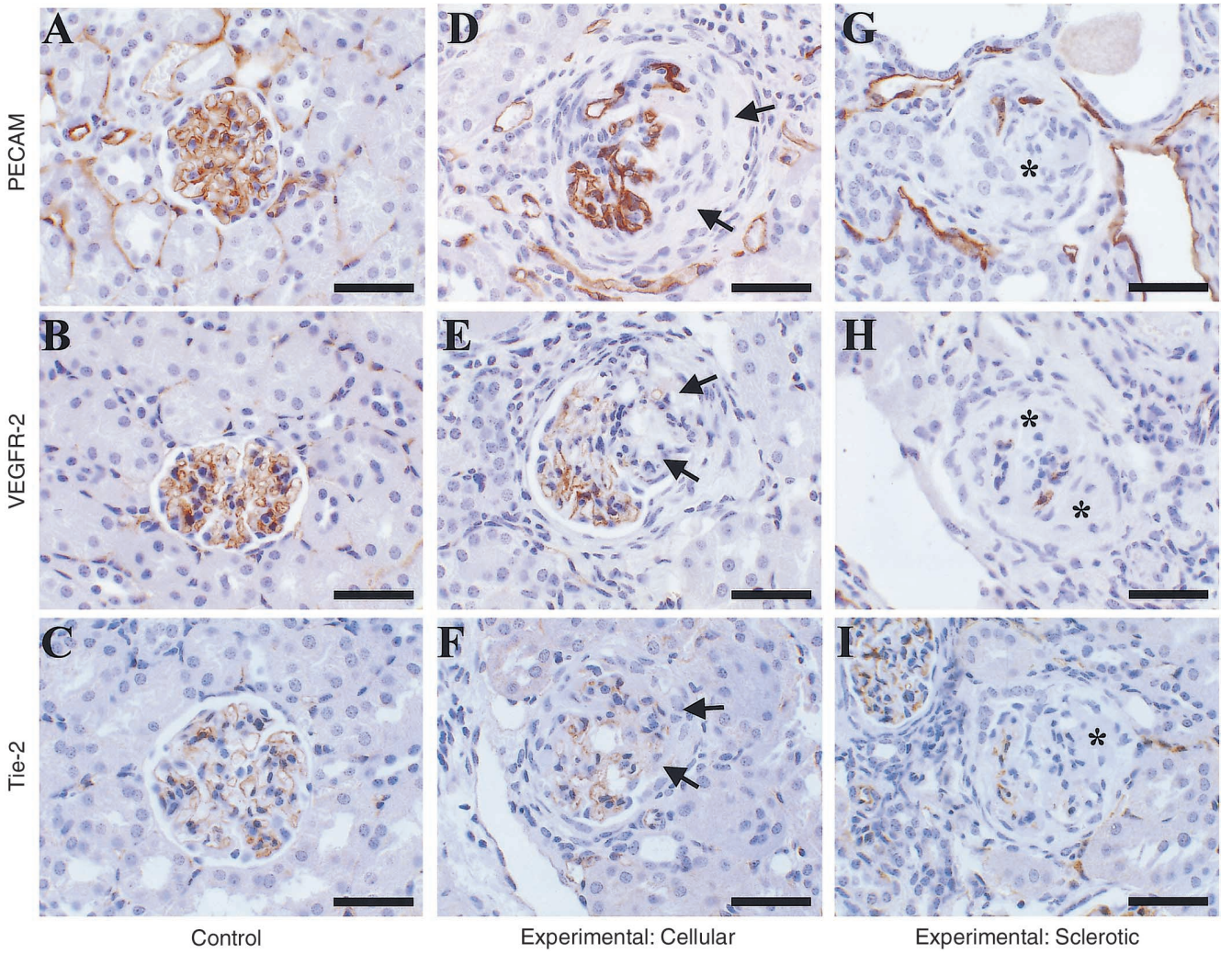

Fig. 3. PECAM, VEGFR-2, and Tie-2 immunohistochemistry. Sections stained with hematoxylin. (*A, D* and *G*) Reacted with PECAM antibody. (*B, E* and *H*) Reacted with VEGFR-2 antibody. (*C, F* and *I*) Reacted with Tie-2 antibody. (A-C) In control glomeruli, capillaries are immunoreactive for PECAM, VEGFR-2 and Tie-2. (D-F) In sections from anti-GBM GN kidneys, immunostaining for these endothelial markers is clearly diminished in cellular lesions (arrows). (G-I) In sections from anti-GBM GN kidneys, endothelial immunostaining is almost absent in sclerotic glomeruli (*). Bars are 80 μm.

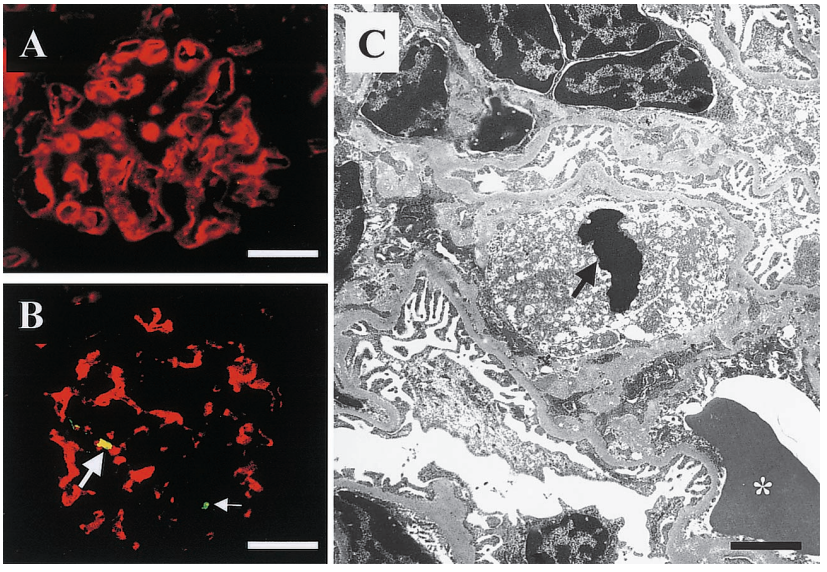

Fig. 4. Detection of apoptosis in glomeruli. (*A* and *B*) Immunostained with PECAM antibody and a Texas red second antibody; additionally, they were probed with the TUNEL kit, with apoptotic nuclei appearing green. (A) Glomerulus from control kidney shows no apoptosis in capillaries that express PECAM. (B) Two apoptotic nuclei are noted in this glomerulus affected by a segmental lesion in an anti-GBM GN kidney. One (large arrow) is double-labeled and appears yellow and hence is a dying endothelial cell; the other (small arrow) is green and hence is a non-endothelial apoptotic cell. (*C*) Electron microscopic view of a GN glomerulus showing a nucleus (arrow) in a capillary lumen; it has the condensed and dense features of an apoptotic body. An erythrocyte is indicated (*) in an adjacent capillary. Bars are 40 μm in A and B, and 0.5 μm in C.

Table 1. Apoptotic endothelial cells in glomeruli

Group	Total Gl	Gl with TUNEL +ve cells	Gl with TUNEL and PECAM double +ve cells	Gl with TUNEL +ve cells which were PECAM −ve
		%		
Control	212 ± 13	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
E14	198 ± 14	5.3 ± 0.5[a]	1.0 ± 0.2[a]	4.9 ± 0.4[a]
E21	224 ± 12	6.8 ± 0.3[a]	1.2 ± 0.2[a]	6.1 ± 0.4[a]

Group data are given as mean ± SEM ($N = 4$) in each group. Abbreviations are: Gl, glomeruli; E14, experimental day 14; E21, experimental day 21; TUNEL, terminal deoxy transferase uridine triphosphate nick end-labeling; PECAM, platelet-endothelial cell adhesion molecule; +ve, positive; −ve, negative.

[a] $P < 0.01$ in experimental versus control groups

ers was reduced within focal GN lesions, with fewer or even absence of capillary loops: Figure 3 panels D to F respectively show PECAM, VEGFR-2 and Tie-2 in glomeruli with cellular lesions, while Figure 3 panels G to I depict glomeruli affected by sclerosis where the sections have been probed with the same group of antibodies. We considered that some capillaries might have been 'lost' through the process of endothelial apoptosis. Quantification of cell death revealed that 8.9 ± 0.7% glomeruli contained one or more apoptotic nuclei on experimental day 14, while on experimental day 21, 11.4 ± 1.1% contained at least one apoptotic cell, with no significant difference between the counts at 14 and 21 days. This showed that apoptosis was occurring but not which cells were undergoing programmed death. Using PECAM immunostaining and in situ end-labeling in the same sections, apoptotic glomerular endothelial cells were not observed in kidneys of control mice (Fig. 4A). In contrast, a subset of apoptotic cells in glomeruli from the experimental groups expressed PECAM (Fig. 4B), and an electron microscope survey of glomeruli from experimental animals revealed rare apoptotic nuclei attached to capillaries walls or within their lumens (Fig. 4C). On further inspection (Table 1), it was noted that about 1% of all glomeruli in both experimental groups contained endothelial cells that were apoptotic; only rarely did any glomerular cross-section contain more than one such cell (data not shown). By contrast, control glomeruli did not contain apoptotic endothelia (Table 1). Glomerular cross-sections containing one or more non-endothelial apoptotic cells (that is, cells that were TUNEL-positive but PECAM-negative) were more common in the experimental groups (~5 to 6% of all glomeruli counted) versus the controls (0.2% of all glomeruli counted; Table 1).

Ang-1, Ang-2 and VEGF immunohistochemistry

In control kidneys, Ang-1 immunostaining was noted in all glomeruli, in a pattern consistent with capillary staining (Fig. 5A). Ang-1 protein also was detected in a capillary-like pattern between cortical tubules that were negative themselves (Fig. 5A). In glomeruli from control kidneys, absent or minimal glomerular Ang-2 immunostaining was noted (Fig. 5B). VEGF-A immunoreactivity was detected in control glomeruli (Fig. 5C), in a podocyte-like pattern, as described by Brown et al [31]. At 14 days after administration of anti-GBM globulin, different patterns were noted for all three factors. First, all glomeruli, whether or not affected by morphological evidence of GN, had decreased Ang-1 immunostaining (Fig. 5D). Prominent Ang-1 immunostaining instead was detected in a subset of narrow cortical tubules that were often located next to glomeruli (Fig. 6). This is consistent with them being at the top of the ascending limb of loop of Henle and/or at the start of the distal convoluted tubule, a conclusion supported by the lack of prominent brush border, as assessed by PAS staining. Ang-2 immunoreactivity was evident at 14 days after induction of GN within the tufts of glomeruli (Fig. 5E). VEGF-A immunoreactive protein appeared less prominent than in controls in glomeruli affected by cellular crescents (Fig. 5F). In glomeruli affected by sclerosis (Fig. 5 G-I), a broadly similar pattern (that is, decreased Ang-1, increased Ang-2 and decreased VEGF-A) was noted. Vascular growth factor proteins also were detected in renal arteries (data not shown). Ang-1 was noted in arterial endothelia in controls, while Ang-2 and VEGF immunostaining was prominent in the muscular layers of renal arteries from controls; after induction of GN, immunostaining for all three factors was maintained, with prominent vascular wall immunoreactivity for all three proteins.

Northern and Western blotting for Ang-1, Ang-2 and VEGF-A

Having obtained the immunohistochemical data, described above, we attempted to quantify the expression of these growth factors using Northern and Western blots of kidney cortex. Transcripts for Ang-1 (4.8 kb), Ang-2 (2.8 kb) and VEGF-A (3.9 kb) were detected in control kidneys and organs at 14 and 21 days after induction of anti-GBM GN (Fig. 7A). Mean mRNA levels of these genes, factored for 18S RNA, are shown in Figure 7B. Levels of transcripts for all three growth factors tended to increase at experimental day 14 versus untreated controls, and this was statistically significant for the angio-

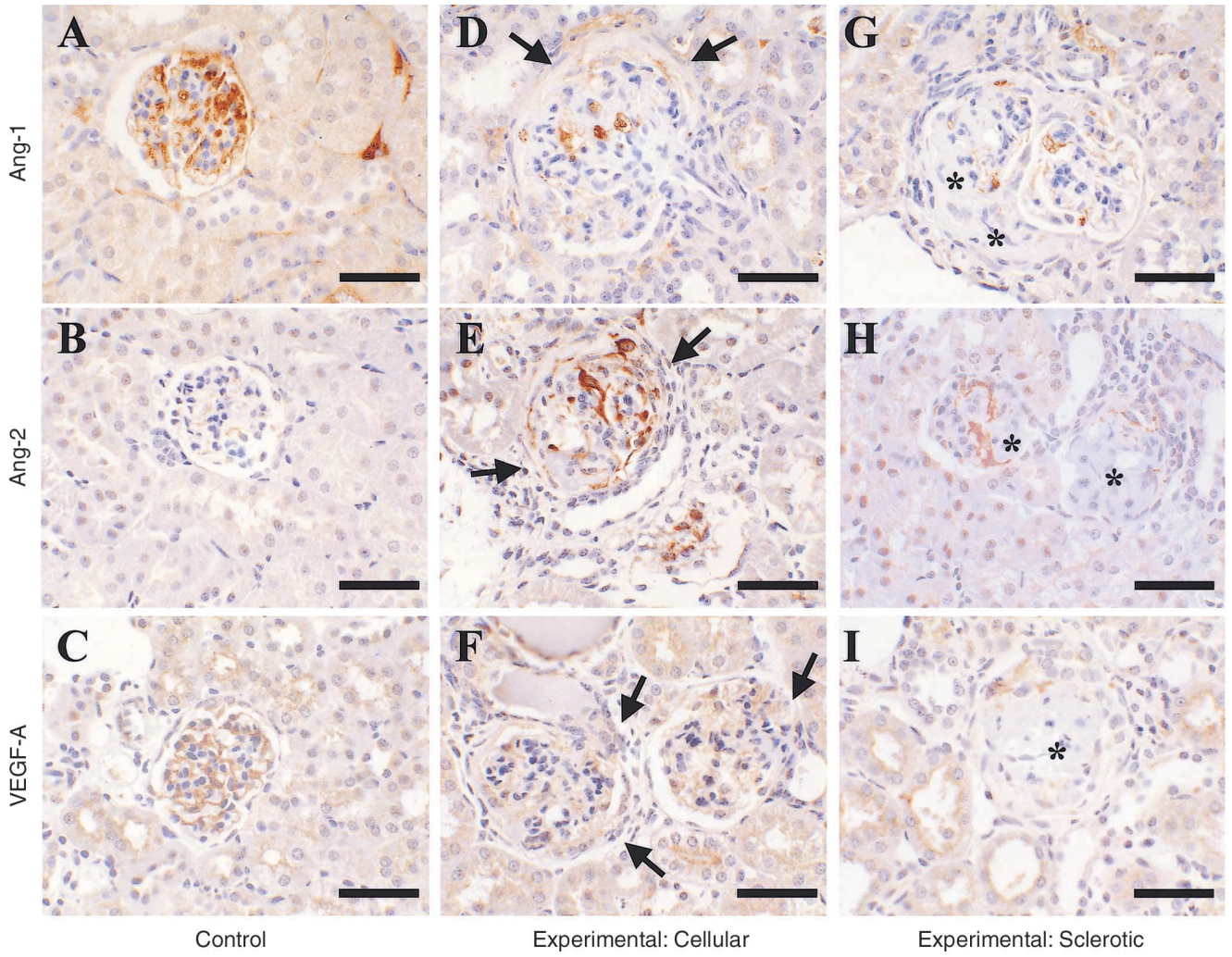

Fig. 5. Ang-1, Ang-2 and VEGF-A immunohistochemistry of glomeruli. All sections were stained with hematoxylin. (*A, D* and *G*) Reacted with antibody to Ang-1. (*B, E* and *H*) Reacted with antibody to Ang-2. (*C, F* and *I*) Reacted with VEGF-A antibody. (A-C) In sections from control kidneys, glomerular capillary loops contain immunoreactive Ang-1 (A) and VEGF-A (C), whereas Ang-2 is barely detectable (B). (D-F) These are sections from anti-GBM GN kidneys showing cellular lesions (arrows). Ang-1 immunostaining is diminished in glomerular tufts (D), Ang-2 immunostaining is detectable in affected glomeruli (E), and VEGF-A immunostaining appears diminished in affected glomerular tufts (F). (G-I) In sections from anti-GBM GN kidneys showing sclerotic glomerular lesions (*), immunostaining for Ang-1 (G) and VEGF-A (I) were decreased versus normals, whereas Ang-2 protein is detectable is some sclerotic glomeruli (H). Bars are 100 μm.

poietins. On experimental day 21, Ang-1 and VEGF-A levels were not significantly different to controls, whereas Ang-2 mRNA remained elevated. As assessed by Western blot, Ang-1, Ang-2 and VEGF-A signals were detected in all control and experimental kidney samples (Fig. 8A), and these signals were abolished after prereacting primary antibodies with relevant immunizing peptides (data not shown). Ang-1 bands were observed at 50 kD, probably representing the monomer, and at 100 kD, most likely a dimer. The Ang-2 Western blot showed

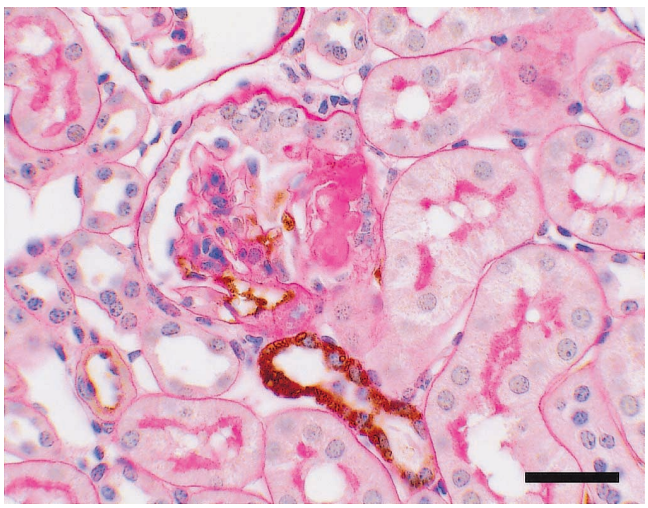

Fig. 6. Ang-1 immunostaining in distal tubule. The section from a two-week experimental kidney was probed with Ang-1 antibody and counterstained with hematoxylin and PAS. Note that there is little Ang-1 expression in the glomerular lesion, whereas there is prominent immunostaining in an adjacent thin tubule, which lacks a PAS-positive brush border. This is likely to be a distal tubule. Bar is 40 μm.

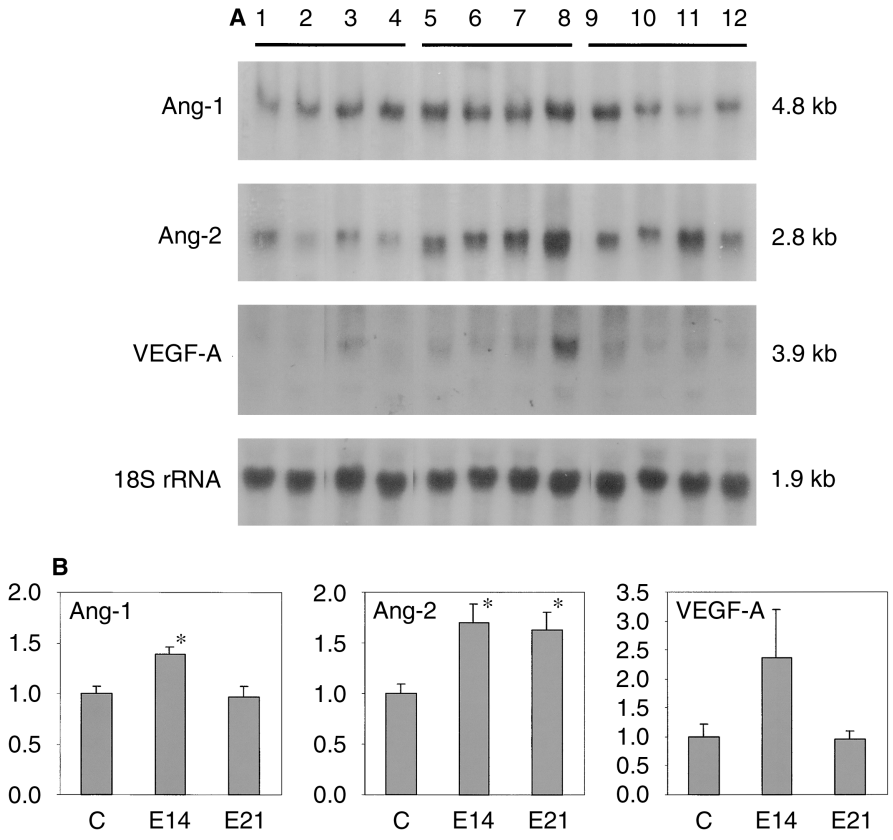

Fig. 7. Northern blotting. (*A*) Northern blotting for Ang-1, Ang-2, VEGF-A and 18S mRNA. Note expression of these species in kidneys from four controls (lanes 1-4) and 14 days (lanes 5-8) and 21 days (lanes 9-12) after induction of anti-GBM GN. (*B*) The intensities of the Ang-1, Ang-2 and VEGF-A signals were factored for 18S rRNA, and the results expressed as bar charts ($N = 4$ for each experimental group). The mean intensity of the control groups was designated as an arbitrary value of 1. Levels of Ang-1 were significantly increased at day experimental 14 versus controls, while those for Ang-2 were significantly increased at experimental day 14 and 21. Abbreviations are: C, controls; E14, experimental day 14; E21, experimental day 21; $*P < 0.05$ versus controls.

major bands at 50 kD and 75 kD, probably representing the monomer and a glycosylated form (Alpha Diagnostic International data sheet). On the VEGF-A Western blot, a 22 kD was observed, representing a monomer. Using densitometry (in the cases of Ang-1 and Ang-2, summing the signal intensity of the two main bands), a statistically significant increase of Ang-1 protein was found at 14 ($P < 0.05$) and 21 days ($P < 0.01$) after administration of anti-GBM globulin (Fig. 8B). Mean Ang-2 levels increased during the course of GN, and this was significant at day 21 ($P < 0.05$). VEGF-A was significantly ($P < 0.05$) increased versus controls at experimental day 14.

DISCUSSION

Anti-glomerular basement membrane induced GN is a model of glomerular injury in mice that results from an immune initiated, leukocyte-mediated inflammatory response directed against an antigen (sheep globulin) planted on the glomerular basement membrane. In C57BL/6 mice, the immune response is predominantly of a Th1 type [32] and requires MHC II dependent antigen presentation [33] and CD4 T cells [29]. This results in a proliferative and crescentic GN, characterized by glomerular accumulation of macrophages, proliferation of intrinsic glomerular cells [34] and fibrin deposition. Glomerular sclerosis, interstitial inflammation and renal impairment develop secondarily to the glomerular inflammatory response. Local fibrin deposition contributes to crescent formation, sclerosis and renal impairment [35] and plasminogen activators produced by endothelial cells and leukocytes play an important role in regulating this process [36].

While many studies have pointed to inflammatory cells and glomerular epithelia as being important in the biology of experimental crescentic GN [37], it is becoming apparent that the fate of glomeruli in several types of GN may be associated with the turnover of capillaries. Furthermore, although it is probable that some capillaries are lost by necrosis, especially in the more acute models of disease, endothelial apoptosis may be correlated with outcome. Endothelial injury and regeneration take place respectively during Habu snake venom-induced GN [38] and anti-Thy 1 antibody-induced GN [39] in rats; the latter process is accompanied by upregulated glomerular VEGF expression. Moreover, antagonism of VEGF activity in the latter model causes a disturbance of capillary remodelling and endothelial apoptosis [40]. In other models of glomerular disease, characterized by failure of glomerular morphological recovery, an overall loss of glomerular endothelia takes place. In five-sixth nephrectomized rats, Kitamura et al reported

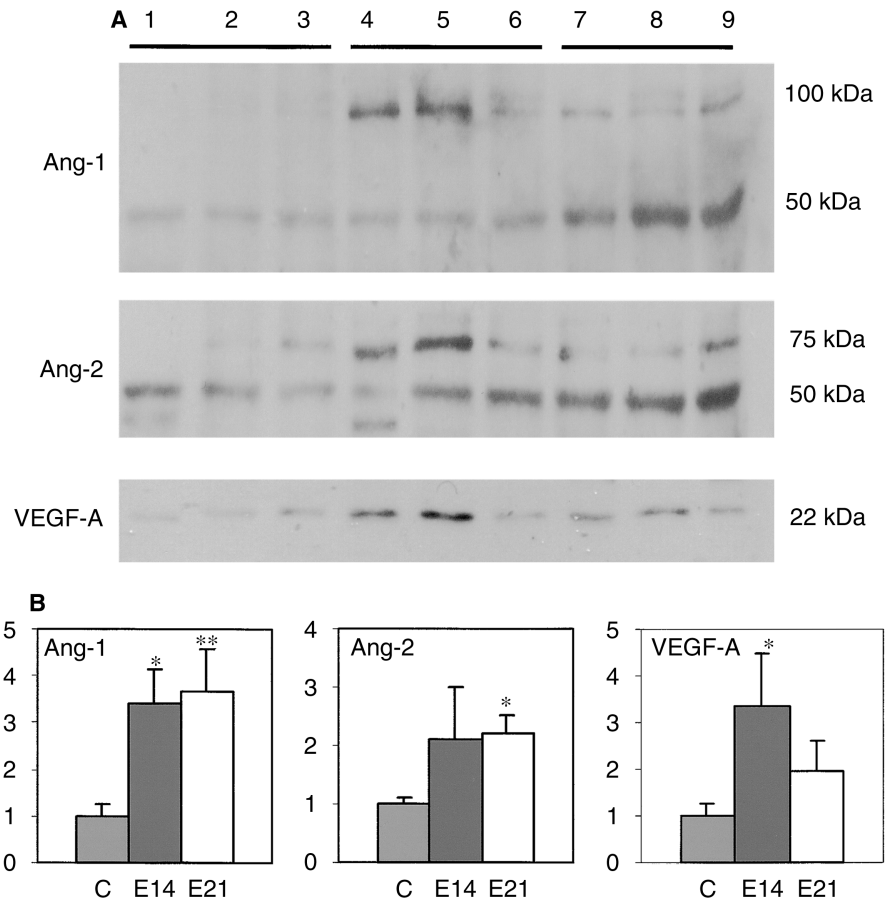

Fig. 8. Western blotting. (*A*) Western blots for Ang-1, Ang-2, VEGF-A. Note expression of these proteins in three kidney cortex samples from controls (lanes 1-3) and at 14 days (lanes 4-6) and 21 days (lanes 7-9) after induction of anti-GBM GN. (*B*) Intensities of the Ang-1, Ang-2 and VEGF-A signals were factored for G Brilliant blue staining and results expressed as bar charts ($N = 3$ for each experimental group); for Ang-1 and Ang-2, the two major protein species were summated. The mean intensity of the control groups was designated as an arbitrary value of 1. Levels of Ang-1 were significantly increased at experimental days 14 and 21 versus controls. Ang-2 levels were significantly increased at experimental day 21. VEGF-A was significantly increased at experimental day 14. Abbreviations are: C, controls; E14, experimental day 14; E21, experimental day 21; $*P < 0.05$ and $**P < 0.01$ versus control.

that diffuse glomerulosclerosis occurred at three-four months, with significant apoptotic deletion of glomerular endothelia, detected by expression thrombomodulin, an endotheila surface glycoprotein, at three months [23]. Shimuzu et al studied anti-GBM GN in rat after a single injection of anti-rat GBM anybody, and found an overall loss of glomerular endothelia over several weeks, with an early increase of proliferation followed by apoptotic deletion of these cells, as assessed by colocalization of TUNEL with thrombomodulin [24]. Masuda et al combined Habu-venom and Thy 1 antibody administration to produce a severe lesion with acute destruction of the glomerular capillary network [41]; despite an up-regulation of glomerular VEGF, endothelial regeneration was rare and the kidneys proceeded to glomerulosclerosis unless rescued by exogenous VEGF administration. Finally, in a model of glomerular thrombotic microangiopathy, Kim et al reported that VEGF administration enhanced glomerular regeneration [26].

The results presented in the current study add a new perspective to the biology of anti-GBM GN, in terms of vascular growth factor expression, especially in relation to the deletion of glomerular endothelia, a process at least partly mediated by apoptosis. The Northern and Western blotting results demonstrate that levels of Ang-1, Ang-2 and VEGF-A in lysates of kidney cortex tended to increase by day 14 after administration of anti-GBM globulin. At experimental day 21, Ang-2 levels remain elevated, whereas VEGF-A was the same as controls. When expression was studied by immunohistochemistry, however, a more complex pattern emerged, with some factors expressed in glomeruli, tubules and arteries. Renal arteries expressed Ang-1, Ang-2 and VEGF-A, and therefore protein in these structures should be taken into account when interpreting Western blotting using lysates of kidney cortex. Both VEGF-A and Ang-1 protein were detectable in control glomeruli. The pattern for VEGF-A is compatible with a predominantly podocyte localization, as reported by Brown et al [31], although we cannot exclude lower protein levels in other intrinsic cells. In addition, Ang-1 immunostaining was evident in control glomeruli. While it is difficult to discern exactly which cells contain this protein, we have previously used RT-PCR on mouse mesangial cells demonstrating that they do not express Ang-1 in culture [22], while other experiments in developing mouse kidneys suggested that Ang-1 transcripts are expressed by podocytes [20]. In this study, we did not detect significant

Ang-2 expression in control mouse glomeruli; indeed, our previous studies showed that Ang-2 is expressed by mesangial cells during mouse glomerulogenesis and that it is down-regulated in adulthood [21, 22]. While the exact roles, if any, of either VEGF-A or Ang-1 in the healthy adult glomerulus are unknown, it is possible to speculate that they both enhance endothelial survival. Certainly, both factors have this action on cultured endothelia via the PI3K/Akt pathway [42]. Alternatively, Ang-1 may act to maintain integrity between glomerular endothelia themselves and between these cells and supporting pericytes, as outlined at the beginning of this article. A low level of Tie-2 tyrosine phosphorylation can be detecting in the adult kidney [20], supporting a possible role for Ang-1. Proof of these hypotheses would require in vivo blockade of both these factors in normal adult mice.

After induction of anti-GBM GN, we found marked changes in these patterns in glomeruli. First, Ang-1 immunolocalization was almost completely lost in glomeruli, whether or not they were affected by histological proliferative and/or sclerotic lesions. At first we found this extensive pattern surprising; however, as assessed by P-selectin up-regulation, we also found that glomerular endothelial 'activation' was more extensive than the subset of glomeruli affected by gross morphological lesions. Instead of the normal glomerular expression of Ang-1, a new pattern became apparent at two weeks after disease induction, with the factor immunolocalized to cortical distal tubules, a site where protein could not be detected in controls. Isbel et al have observed that tubular macrophage colony stimulating factor is up-regulated in an accelerated model of anti-GBM GN, possibly by cytokines released by inflamed glomeruli [43], and we speculate that a similar mechanism may apply to Ang-1 induction. Interestingly, Long et al found that distal convoluted tubules expressed Ang-1 protein after folic acid-induced nephropathy in mice [25], and our preliminary data show that a mouse distal tubule line can express Ang-1 (abstract; Long et al, *J Am Soc Nephrol* 12:656A, 2001). In addition, in our current model, Ang-2 became detectable in glomeruli affected by histological proliferative and/or sclerotic lesions. Previous work has shown that mesangial cells express Ang-2 during glomerulogenesis in vivo and that cultured mesangial cells from normal adult mice up-regulate Ang-2 transcripts and protein expression upon exposure to hypoxia [21, 22]; other evidience suggests that Ang-2 expression by endothelia can be modulated by inflammation [44]. Some of these factors may be operative in glomerular Ang-2 up-regulation in anti-GBM GN.

The biological effects of angiopoietins most likely depend on the interplay between Ang-1 and Ang-2 themselves, with respect to Tie-2 activation and VEGF signaling. In vitro, Ang-1 and VEGF synergize to induce capillary sprouting [45]. Furthermore, with abundant VEGF, Ang-2 disrupts vessels, thus facilitating sprouting; conversely, when ambient VEGF is low, Ang-2 is associated with vessel regression [16, 18]. In glomeruli affected by histological lesions, we observed that the distinct pattern of VEGF-A immunostaining evident in controls was lost, and was replaced by a low level of diffuse immunoreactivity. We suggest that this changed glomerular pattern of vascular growth factors (that is, increased Ang-2 with decreased Ang-1 and VEGF) tends to be anti-angiogenic, and could enhance endothelial apoptosis. Indeed, we could not detect apoptotic endothelia in control glomeruli, either by electron microscopy or in situ end-labeling, but such cells were evident in experimental glomeruli. Although such apoptotic endothelial cells were relatively rare (noted in ~1% of all glomerular cross-sections in the experimental groups), they could have important biological implications, especially if the rate of their clearance was rapid. Furthermore, there was a loss of glomerular endothelia in this model, with sclerotic lesions containing few, if any, cells reactive with either PECAM, VEGFR-2 or Tie-2 antibodies. This type of change in the balance of several vascular growth factors is thought to determine angiogenesis and vessel regression during physiological states such as corpus luteum growth and involution [16, 18], and in diseases such as impaired cutaneous wound healing in diabetic mice [46].

However, it is probable that glomerular cell survival is affected by a multiplicity of factors, especially in disease states. Based on studies in other systems, pro-apoptotic stimuli include other secreted factors (such as angiostatin, endostatin, transforming growth factor β1, tumor necrosis factor) and physical factors such as intracellular acidosis and ischemia/reperfusion, while anti-apoptotic stimuli include fibroblast and hepatocyte growth factors and extracellular matrix components [47]; some of these also may be operative in anti-GBM GN. In future, the significance of each factor will need to be explored by functional experiments. It will be of special interest to see whether Ang-1 administration can prevent glomerular endothelial loss in anti-GBM GN, since the factor enhances the appearance of mouse metanephric glomerular capillary loops in vitro [4] and augments revascularization of rabbit ischemic hindlimbs [48], while transgenic overexpression in mice also increases tissue vascularization [49].

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